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(71) Applicant *(for all designated States except US)*: HYBRIDON, INC. [US/US]; One Innovation Drive, Worcester, MA 01605 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: ZHANG, Zhaoda [CN/US]; 60 Commons Drive #43, Shrewsbury, MA 01545 (US). TANG, Jimmy, X. [CN/US]; 40 Commons Drive #20, Shrewsbury, MA 01545 (US). TANG, Jin, Yan [CN/US]; 19 Sheridan Drive, Shrewsbury, MA 01545 (US).

(74) Agent: GREENFIELD, Michael, S.; Banner & Allegretti, Ltd., Ten South Wacker Drive, Chicago, IL 60606 (US).

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(57) Abstract

Antisense oligonucleotides having improved cellular uptake, increased nuclease resistance, and thermodynamically more stable target-binding capacity are disclosed. These novel oligonucleotides are characterized by having from 1 to 10 thiono-triester phosphorothioate internucleoside linkages bearing lipophilic moieties.

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THIONO TRIESTER MODIFIED ANTISENSE OLIGODEOXYNUCLEOTIDE PHOSPHOROTHIOATES

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to the field of modified oligodeoxynucleotides, methods for their synthesis, and methods of their use to inhibit gene expression.

Summary of the Related Art

Antisense oligonucleotides and their modified analogs have been shown to regulate the expression of genes (Zamecnik in *Prospects for Antisense Nucleic Acid Therapy for Cancer and AIDS*, pp. 1-6 (Wickstrom, E, Ed.,Wiley Liss, N.Y., 1991); Agrawal, *Trends in Biotechnology* **10**, 152-158 (1992); Wickstom, *Trends in Biotechnology* **10**, 281-286 (1992); Rapaport et al., *Proc. Natl. Acad. Sci.* USA **89**, 8577-8580 (1992); and Agrawal, S. (1991) in *Prospects for Antisense Nucleic Acid Therapy for Cancer and AIDS*, pp. 145-158, *supra)*) and to be effective inhibitors of HIV (Agrawal et al,. *Proc. Natl. Acad. Sci. USA* **85**, 7079-7083 (1988); Agrawal et al., *Proc. Natl. Acad. Sci. USA* **86**, 7790-7794 (1989); Agrawal and Sarin in *Advanced Drug Dilivery Reviews*, pp. 251-270 (Juliano, R. J., Ed., Elsevier, Amsterdam 1991); Agrawal and Tang, Antisense Res. Dev. **4**, 261-266 (1992); Liziewize et al., Proc. Natl. Acad. Sci. USA **89**, 11209-11213 (1992); and Liziewize et al., *Proc. Natl. Acad. Sci. USA* **90**, 3860-3864 (1992)), influenza virus (Leiter et al., *Proc. Natl. Acad. Sci. USA* **87**, 3430-334 (1990)), human papilloma virus (Vickers, et al., *Nucleic Acids Res.* **19**, 3359-3368 (1991)) and herpes simplex virus (Gao et al., *Antimicrob. Agents Chem.* **34**, 808-812 (1990)) in tissue culture studies. To use antisense oligonucleotides as drugs it is necessary to develop compounds that are stable under biological and physiological conditions, that are capable of effectively binding to a complimentary nucleic acid target, and that can be readily taken up by cells.

To meet these criteria, considerable efforts have been made recently in the structure modification of oligonucleotides (Uhlmann and Peyman, *Chem. Rev.* **90**, 543-584 (1990) and references cited therein; *Methods in Molecular Biology Vol. 20: Protocols for Oligonucleotides and Analogs* (Agrawal, S., Humana Press, New Jersey, 1993) and references cited therein; *Antisense Research and Applications* (Crooke, S.T. and Lebleu, B., Eds., CRC press, Ann Arbor 1993) and references cited therein), including modifications of the internucleoside linkages such as phosphorothioates, methylphosphonates, phosphoramidates, phosphotriesters (Koziolkiewicz

and Wilk, in *Protocols for Oligonucleotides and Analogs Vol. 20: Protocols for Oligonucleotides and Analogs*, pp. 207-224, *supra*, and references cited therein), and many other non-phosphat internucleoside linkages.

Miller et al., *Biochem.* **21**, 5468 (1982), disclosed both isomeric forms of a decamer having an internal ethylphosphotriester linkage. It was found that after adding polyadenylate tails to the 3' end of the decamers, the ethyl triester group inhibited polymerization by *E. coli* DNA polymerase I.

Stec et al., *Tetrahedron Lett.* **26**, 2191 (1985), disclosed dimers linked by an isopropyl phosphorothioate triester group. These dimers were synthesized via a 3'-O-isopropyl phosphomorpholidite intermediate.

Hau et al., *Tetrahedron Lett.* **32**, 2497 (1991), discloses thiolation of O-neopentylphosphite dimer using elemental sulfur (S_8) in pyridine to yield the corresponding neopentylphosphorothioate triester dimer. Four of these dimers were then linked via phosphodiester linkages to yield an 3'-acridine-capped octamer having alternating phosphodiester and neopentyl phosphorothioate triester linkages.

Letsinger et al., *Proc. Natl. Acad. Sci. USA* **86**, 6553 (1989), disclosed oligonucleotides having one 3'-terminal cholesteryl phosphorothioate triester internucleoside linkage. They observed inhibition of syncytia formation and expression of viral proteins p17, p24, and reverse transcriptase of HIV I in Molt-3 cells. The best results were found with an eicosomer, although decamers and a heptanucleotide where also active. These results, combined with the observation that the activity of the decamers did not correlate with a specific sequence, suggested that a mechanism other than antisense inhibition may be operative.

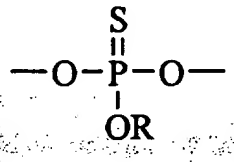
Letsinger et al., *Nucleic Acids Res.* **14**, 3487 (1986), synthesized nucleotide dimers and a trimer with O-phosphotriester and phosphoramidate internucleotide linkages bearing C_2-C_4 trichloroalkyl moieties. They found that these dimers and trimers bound to target polynucleotides relatively stably.

Enzymatic degradation of oligonucleotide phosphorothioates is primarily from the 3' end. In an effort to circumvent this problem, various modifications at the oligonucleotide phosphorothioate 3' end have been made. Koziolkiewicz et al., *supra*; Temsamani et al., *Antisense Res. Dev.* **3**, 277 (1993); Mackelar, et al., *Nucleic Acids Res.* **20**, 3411 (1992); and Tang et al., *Nucleic Acids Res.* **21**, 2729 (1993).

The foregoing demonstrates the continuing interest in developing antisense olig nucleotides with increased efficacy for hybridizing to target nucleic acids. Such oligonucleotides exhibit strong cellular uptake, high stability when bound to the target, and high resistance to nuclease attack.

SUMMARY OF THE INVENTION

The present invention comprises a novel class of modified antisense oligonucleotides having increased cellular uptake, high stability when bound to the target nucleic acid, and high resistance to nuclease attack. These novel antisense oligonucleotides are characterized by having one to ten thiono triester phosphorothioate internucleotide linkages. These internucleotide linkages have the following structure:



where R is a large lipophilic moiety, such as a straight or branched chain alkyl group, a cholesteryl derivative, or an adamantyl derivative. Oligonucleotides having one or more of these types of linkages exhibit increased resistance to degradation by T4 polymerase and DNA polymerase I. They also exhibit increased melting temperatures when hybridized to target nucleic acids as compared to oligonucleotide phosphorothioates.

The oligonucleotides of the present invention are useful for both *in vitro* and *in vivo* suppression of nucleic acid expression. Among the *in vitro* uses for the present oligonucleotides is the determination of the role particular proteins play in biological processes by modulating the expression of the genes encoding the protein under study. The elucidation of most biochemical pathways now known was accomplished by isolating and studying deletion mutants *in vitro*. Studying deletion mutants is arduous, however. The presently claimed oligonucleotides provide an attractive alternative because it is much less laborious to modulate gene expression using antisense oligonucleotides than it is using the deletion mutation approach. Thus, the oligonucleotides of the present invention are useful research tools.

The oligonucleotides of the present invention are suitable for *in vivo* use as well. The increased stability of the oligonucleotide-target hybridization complex, the increased resistance to nuclease attack, and the increased susceptibility to cellular uptake all make the oligonucleotides

of the present invention ideal for treating infections by a wide variety of diseases caused by pathogens, including viral and bacterial agents. When designed for *in vivo* use, the presently disclosed oligonucleotides will have at least one region whose sequence is sufficiently complementary to a region of the pathogen's nucleic acid (the target nucleic acid) to result in hybridization to the target nucleic acid and suppression of its expression, both under intracellular conditions.

Because of their efficacy at gene modulation, the presently claimed oligonucleotides are also useful for treating diseases arising from genetic abnormalities that cause under- or over-expression of a gene. For diseases in which an abnormal gene is over-expressed, for example, the presently claimed oligonucleotides may be designed to target the abnormal gene directly, or, in the alternative, to target the gene encoding the protein that promotes expression of the abnormal gene. Conversely, where an abnormal gene is under-expressed, one may design an oligonucleotide that suppresses expression of a gene encoding a protein that suppresses expression of the abnormal gene.

To summarize, the presently claimed oligonucleotides are useful both *in vitro* and *in vivo* in essentially any situation in which one desires to modulate gene expression.

The present invention also provides novel compositions comprising the inventive oligonucleotides as well as methods for employing the oligonucleotides to treat pathogenic diseases and other abnormal states arising from aberrant gene expression.

The foregoing merely summarizes certain aspects of the present invention and is not intended, not should it be construed, to limit the invention in any manner. All patents and other publications referred to in this specification are hereby incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 displays the synthetic scheme for producing phosphoramidite intermediates useful in the production of oligonucleotides bearing S-triester internucleotide linkages.

Figure 2 displays an autoradiogram of the digestion of modified S-triester-phosphorothioate oligonucleotides by T4 polymerase.

Figure 3 displays an autoradiogram of the digestion of an *O*-ethyl triester phosphorothioate by DNA polymerase I.

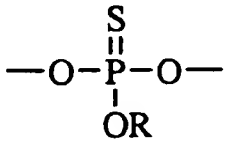
Figure 4 displays an autoradiogram of the digestion of an ordinary phosphorothioate and an *O*-ethyl triester phosphorothioate oligonucleotide by T4 polymerase.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

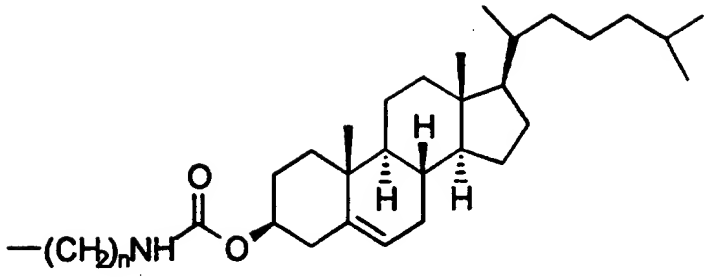
The present invention comprises, *inter alia*, a new class of antisense oligonucleotides having improved properties relative to prior art antisense oligonucleotides. The present oligonucleotides exhibit increased susceptibility to cellular uptake when carrying a large lipophilic group, increased resistance to exonucleolytic degradation, and increased stability of hybridization complexes formed between the oligonucleotides and their targets. These properties make these novel oligonucleotides ideal for modulating gene expression, both *in vitro* and *in vivo*.

Oligonucleotides according to the invention may be anywhere from 2 to 100 nucleotides in length. In a preferred embodiment, the oligonucleotides will be from 13 to 50 nucleotides in length. In a more preferred embodiment, the oligonucleotides will be from 20 to 35 nucleotides in length.

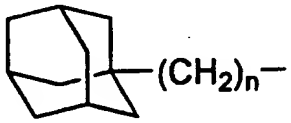
In one embodiment, the present invention comprises antisense oligonucleotides having one to ten thiono triester containing oligodeoxynucleotide phosphorothioate (S-triester-phosphorothioate) internucleotide linkages having the following structure:



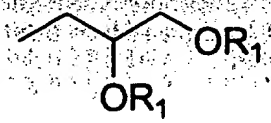
where R is any large lipophilic group that doesn't substantially change the geometry of the internucleotide bond in a manner that dimishes the oligonucleotide's efficacy at modulating gene expression. In a preferred embodiment, R is a C_2-C_{22} linear or branched chain alkyl group, a cholesteryl derivative having the structure:



where n = 2-12, an adamantyl derivative having the structure:

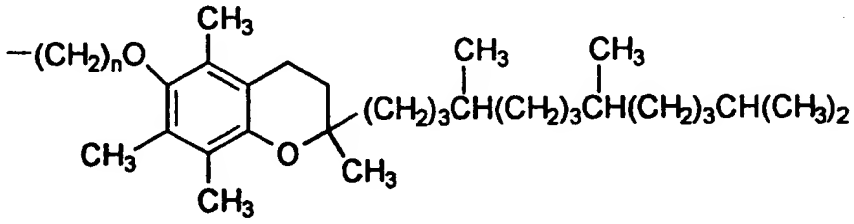


where n = 2-12, a 1,2-di-*O*-alkyl-*rac*-3-glyceryl derivative having the structure:



where R_1 is C_nH_{2n+1} and n = 2-22,

or a DL α-tocopherol (Vitamin E) derivative having the structure:

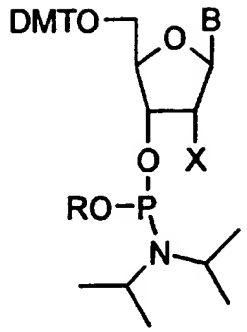


where n = 0-10.

In an oligonucleotide according to the invention having two or more S-triester internucleotide linkages, the R groups of different linkages may be the same or different.

In another preferred embodiment, R is chosen from the group consisting of 1-adamantyl-2-ethyl, 1-hexadecyl, and cholesteryl-3-carboxyamino-6-hexyl.

Oligonucleotides according to the invention are synthesized via a precursor having the following structure:



where R is as described above, B is a suitably protected (if necessary) base, and X is H, F, benzyloxy, $-OCH_2CH=CH_2$, or a linear or branched alkyloxy moiety C_nH_{2n+1}, where n=1-5. Figure 1 displays the scheme for synthesizing these intermediates and representative protocols are provided in the Examples, *infra*.

In one aspect of this embodiment, the oligonucleotide will have from 1 to 10 S-triester phosphorothioate internucleotide linkages. In a preferred embodiment, the oligonucleotides will have an S-triester phosphorothioate internucleotide linkage at the 3' or 5' terminal linkage, or at both the 3' and 5' terminal linkages. Where an oligonucleotide of the invention has more than one S-triester linkage, all of the S-triester linkages may have the same R substituent or they may all be different.

In determining the number of S-triester linkages and the identity of the R group substituents to be incorporated into the oligonucleotides, the artisan will want to consider that increased lipophilicity will result in decreased solubility in aqueous solution and steric interactions may change the conformation and affect hybridization.

By virtue of the new internucleotide linkages disclosed herein, the oligonucleotides of the present invention exhibit increased nuclease resistance and, when having a bulky lipophilic group, exhibit substantially increased cellular uptake relative to the corresponding phosphorothioate internucleotide analogs while still maintaining the ability to specifically hybridize to a complementary target nucleic acid under normal stringency conditions. Increases in melting temperature (T_m) of 5 °C and more have been observed with a corresponding increase in cellular uptake of 2.5 to 3 times that of the phosphorothioate analog. As demonstrated below, it has been found that an increase in melting temperatures is observed even when large R moieties are present, contrary to the expectation that these large substituents might interfere with hybridization.

The antisense oligonucleotides of the present invention may be designed to incorporate a number of additional features that have been demonstrated to increase efficacy. For example, they may be designed to be "self-stabilized," *i.e.*, having a first region sufficiently complementary to a second region to allow for intramolecular hybridization, thereby rendering the oligonucleotide less susceptible to nucleolytic attack. Such oligonucleotides are described in PCT International Application Publication No. WO 94/01550.

Alternatively, the presently disclosed oligonucleotides may be designed to be "fold-back triplex forming," *i.e.*, having a first region complementary to a target nucleic acid and a second region having a sequence that allows for triplex formation by Hoogsteen base pairing between it and the duplex formed by the first region and the target nucleic acid, as described in PCT International Application Publication No. WO 94/17091.

Oligonucleotides according to the invention are useful for both *in vitro* and *in vivo* applications. For *in vitro* applications, the present oligonucleotides are useful as research tools in determining gene function. Because they can be prepared to be complementary to a particular sequence, the present oligonucleotides can be used to selectively inhibit expression of a target gene. The present oligonucleotides thus provide an attractive and easily used alternative to the laborious method of gene inhibition by mutation (*e.g.*, deletion mutation). The significance of this will be appreciated when one realizes that the elucidation of all biological pathways now known was determined by deletion mutations.

The oligonucleotides of the present invention are also useful as therapeutic agents for diseases or physiological conditions involving expression of specific genes. Oligonucleotides useful for treating a disease or condition will have a nucleotide sequence sufficiently complementary to the target nucleic acid to bind under physiological conditions. As used herein, the terms "complementary" and "sufficiently complementary" are used interchangably and, when used to describe the sequence of an antisense oligonucleotide, mean that the oligonucleotide sequence is such that the oligonucleotide inhibits expression of the target nucleic acid under the conditions of interest (*e.g.*, *in vitro* experimental conditions and physiological conditions). In general, oligonucleotides according to the invention will have sequence complementary to a nucleic acid (*e.g.*, a gene or mRNA) that is essential to a biological process. As elaborated more fully below, such processes include reproduction and metabolic processes of pathogens and other disease-causing infectious agents. Or, the biological process can be a naturally occuring one

whose inhibition is desirable, *e.g.*, spermatogenesis in men and ovulation in women desiring contraception. The oligonucleotides of the invention can also be complementary to a gene or other nucleic acid whose expression causes or is involved in a diseased or otherwise abnormal state of the organism.

Because of their efficacy at gene modulation, the presently claimed oligonucleotides are also useful for treating diseases arising from genetic abnormalities that cause under- or over-expression of a gene. For diseases in which an abnormal gene is over-expressed, for example, the presently claimed oligonucleotides may be designed to target the abnormal gene directly, or, in the alternative, to target the gene encoding the protein that promotes expression of the abnormal gene. Conversely, where an abnormal gene is under-expressed, one may design an oligonucleotide that suppresses expression of a gene encoding a protein that suppresses expression of the abnormal gene.

In many cases the target nucleic acid sequence will be a virus nucleic acid sequence. The use of antisense oligonucleotides to inhibit various viruses is well known and has been reviewed in Agrawal, *Tibtech* **10**, 152 (1992). Viral nucleic acid sequences that hybridize to effective antisense oligonucleotides have been described for many viruses, including human immunodeficiency virus type 1 (U.S. Patent No. 4,806,463), Herpes simplex virus (U.S. patent No. 4,689,320), Influenza virus (U.S. Patent No. 5,194,428), and Human papilloma virus (Storey et al., Nucleic Acids Res. 19:4109-4114 (1991)). Sequences hybridizing to any of these nucleic acid sequences can be used, as can nucleotide sequences complementary to nucleic acid sequences from any other virus. Additional viruses that have known nucleic acid sequences against which an antisense oligonucleotide according to the invention can be prepared include, but are not limited to, Foot and Mouth Disease Virus (*See* Robertson et al., *J. Virology* **54**, 651 (1985); Harris et al., *J. Virology* **36**, 659 (1980)), Yellow Fever Virus (*See* Rice et al., *Science* **229**, 726 (1985)), Varicella-Zoster Virus (*See* Davison and Scott, *J. Gen. Virology* **67**, 2279 (1986), Cucumber Mosaic Virus (*See* Richards et al., *Virology* **89**, 395 (1978)), Hepatitis B Virus (*See* Raney and McLachlen, in *Molecular Biology of Hepatitis B Virus* (CRC Press, 1991)), Hepatitis C Virus (*See* Miller and Purcell, *Proc. Natl. Acad. Sci. USA* **87**, 2057 (1990); *Proc. Natl. Acad. Sci. USA* **89**, 4942 (1992); *J. General Virology* **74**, 661 (1993)), and Respitory Syncitial Virus (*See* Collins, in *The Paramyxo Viruses*, Chapter 4, pp. 103-162 (David W. Kingsbury, Ed., 1991)).

Alternatively, the oligonucleotides of the invention can have a nucleotide sequence complementary to a nucleic acid sequence f a pathogenic organism. The nucleic acid sequences of many pathogenic organisms have been described, including the malaria organism, *Plasmodium falciparum*, and many pathogenic bacteria. Examples of pathogenic eukaryotes having known nucleic acid sequences against which oligonucleotides of the present can be prepared include, but are not limited to *Trypanosoma brucei gambiense* and *Leishmania* (*See* Campbell et al., *Nature* **311**, 350 (1984)), and *Fasciola hepatica* (*See* Zurita et al., *Proc. Natl. Acad. Sci. USA* **84**, 2340 (1987)). Antifungal oligonucleotides can be prepared having a nucleotide sequence that is complementary to a nucleic acid sequence from, *e.g.*, the chitin synthetase gene, and antibacterial oligonucleotides according to the invention can be prepared using, *e.g.*, the alanine racemase gene.

In yet another embodiment, the oligonucleotides can have a nucleotide sequence complementary to a cellular gene or gene transcript, the abnormal expression or product of which results in a disease state. The nucleic acid sequences of several such cellular genes have been described, including prion protein (Stahl and Prusiner, *FASEB J.* 5, 2799 (1991)), the amyloid-like protein associated with Alzheimer's disease (U.S. Patent No. 5,015,570), and various well-known oncogenes and proto-oncogenes, such as c-*myb*, c-*myc*, c-*abl*, and n-*ras*.

In addition, oligonucleotides that inhibit the synthesis of structural proteins or enzymes involved largely or exclusively in spermatogenesis, sperm motility, the binding of the sperm to the egg or any other step affecting sperm viability may be used as contraceptives for men. Similarly, contraceptives for women may be oligonucleotides that inhibit production of proteins or enzymes involved in ovulation, fertilization, implantation or in the biosynthesis of hormones involved in those processes. Hypertension can be controlled by oligonucleotides that suppress the synthesis of angiotensin converting enzyme or related enzymes in the renin/angiotensin system; platelet aggregation can be controlled by suppression of the synthesis of enzymes necessary for the synthesis of thromboxane A2 for use in myocardial and cerebral circulatory disorders, infarcts, arteriosclerosis, embolism and thrombosis; deposition of cholesterol in arterial wall can be inhibited by suppression of the synthesis of fatty acyl co-enzyme A: cholesterol acyl transferase in arteriosclerosis; inhibition of the synthesis of cholinephosphotransferase may be useful in hypolipidemia. There are numerous neural disorders in that oligonucleotides of the present invention can be used to reduce or eliminate adverse effects of the disorder. For example,

suppression of the synthesis of monoamine oxidase can be used in Parkinson's disease; suppression of catechol O-methyl transferase can be used to treat depression; and suppression of indole N-methyl transferase can be used in treating schizophrenia. Suppression of selected enzymes in the arachidonic acid cascade (which leads to prostaglandins and leukotrienes) may be useful in the control of platelet aggregation, allergy, inflammation, pain and asthma. Suppression of the protein expressed by the multi-drug resistance (*mdr*) gene, which is responsible for development of resistance to a variety of anti-cancer drugs and is a major impediment in chemotherapy may prove to be beneficial in the treatment of cancer. Nucleotide sequences complementary to nucleic acid sequences from any of these genes can be used for the oligonucleotides according to the invention, as can be oligonucleotide sequences complementary to any other cellular gene or gene transcript, the abnormal expression or product of which results in a disease state.

Antisense regulation of gene expression in plant cells has been described in U.S. Patent No. 5,107,065.

Since the nucleotide sequence of the oligonucleotide can be adapted to form Watson-Crick base pairs with essentially any gene, the therapeutic spectrum of the oligonucleotides of the invention should be very broad. Still, certain diseases are of particular interest. For example, a variety of viral diseases may be treated by oligonucleotides having one or more S-triester phosphorothioates internucleotide linkages, including AIDS, ARC, oral or genital herpes, papilloma warts, flu, foot and mouth disease, yellow fever, chicken pox, shingles, HTLV-leukemia, and hepatitis. Among fungal diseases treatable by oligonucleotides according to the invention are candidiasis, histoplasmosis, cryptococcocis, blastomycosis, aspergillosis, sporotrichosis, chromomycosis, dematophytosis and coccidioidomycosis. The method can also be used to treat rickettsial diseases (*e.g.*, typhus, Rocky Mountain spotted fever), as well as sexually transmitted diseases caused by *Chlamydia trachomatis* or *Lymphogranuloma venereum*. A variety of parasitic diseases can be treated by oligonucleotides of the present invention, including amebiasis, Chegas' disease, toxoplasmosis, pneumocystosis, giardiasis, cryptosporidiosis, trichomoniasis, and *Pneumocystis carini* pneumonia; also worm (helminthic diseases) such as ascariasis, filariasis, trichinosis, schistosomiasis and nematode or cestode infections. Malaria can be treated by oligonucleotides of the present invention, regardless of whether it is caused by *P. falciparum*, *P. vivax*, *P. orale*, or *P. malariae*. The infectious diseases

identified above can all be treated with oligonucle tides having one or more S-triester phosphorothioate linkages because the infectious agents for these diseases are known, and, thus, oligonucleotides according to the invention can be prepared having a nucleotide sequence that hybridizes to a nucleic acid sequence that is an essential nucleic acid sequence for the propagation of the infectious agent, such as an essential gene. As used herein, an essential gene or nucleic acid is one that is required for a biological process and without which the biological process does not occur.

The following examples are provided for illustrative purposes only and are not intended, nor should they be construed, as limiting the invention in any way.

EXAMPLES

The following is applicable to each of the examples presented below, unless indicated otherwise. Anhydrous acetonitrile, tetrahydrofuran, dichloromethane, ethyl alcohol, 2-propanol, pentane, triethylamine, 1-hexadecanol, tetrazole, cholesteryl chloroformate, 6-amino-l-hexanol, bis(diisopropylamino)chlorophosphine and 1-adamantaneethanol were purchased from Aldrich (Milwaukee, WI). Hexane, ethyl acetate, and methanol were purchased from J.T. Baker Inc. (Phillipsburg, N.J.) 5'-DMT-deoxyadenosine (*f*BA) cyanoethyl phosphoramidite, 5'-DMT-deoxycytidine (*f*BA) cyanoethyl phosphoramidite, 5'-DMT-deoxyguanosine (*f*BA) cyanoethyl phosphoramidite, 5'-DMT-thymidine cyanoethyl phosphoramidite, Cap A, Cap B, activator and C-18 SEP-PAK cartridge were purchased from Millipore (Bedford, MA). Beaucage reagent (3H-1,2-benzodithiol-3-one-1,1-dioxide) was purchased from R.I. Chemical (Orange, CA). Fluorescein-ON phosphoramidite was purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). The reagents (Sequagel Sequencing System: concentrate, diluent and buffer) used for PACE were purchased from National Diagnostics (Atlanta, GA). ^{31}P NMR spectra (121.65 MHz) were recorded on a Varian (Palo Alto, CA) UNITY 300 (the chemical shift was correlated to 85% H_3PO_4). Thermal melting data were collected from GBC 920 UV-Vis spectrophotometer (Dandenong, Victoria 3175, Australia). Oligonucleotide synthesis was performed on a 8909 Expedite DNA synthesizer (Mllipore). PAGE was carried out by using Model S2 sequencing gel electrophoresis apparatus (LIFE TECHNOLOGY, Gaithersburg, MD) and EC600-90 power supply (E-C APPARATUS COPORATION, St. Petersburg, FL). Fluorescence was measured by Spectrofluorometer (PTI Technology, South Brunswick, N.J.). The UV absorptance was measured UV-160A UV spectrometer (SHIMADZU, Columbia, MD).

Flow cytometric data was acquired on Epics XL (Coulter, Hialeah, FL). The prepared-HPLC was performed on Waters (Milford, MA) 650E HPLC system with DYNAMX Model UV-C absorpance detector. (Dynamax Instrument Co., Woburn, MA). Flash column chromatography was carried out using silica gel 60 F_{254} (Merck, Gibbstown, N.J.) and t.l.c. on silica-gel 60 F_{254} (Merck). Products were visualized on t.l.c. using either ultraviolet absorption at 264 nm or 5% $(NH_4)_6Mo_7O_{24}$ and 0.2% $Ce(SO_4)_2$ in 5% H_2SO_4 (dark blue color for oxidizable compounds).

Example 1

Synthesis of cholesteryl-3-carboxyamino-6-hexanol

Cholesteryl chloroformate in CH_2Cl_2 (60 ml) was added dropwise at 0 °C to a stirred solution of 6-amino-1-hexanol (2.40 g, 20.5 mmol) and triethylamine (3.6 ml, 2.6 g, 25.6 mmol) in CH_2Cl_2 (30 ml). The mixture was stirred overnight at room temperature. The reaction mixture was filtered to remove the resulting salt. Then 100 ml of CH_2Cl_2 was added To the mixture. The solution was washed with saturated solutions of $NaHCO_3$ and NaCl, dried over anhydrous Na_2SO_4, and filtered. The solvent was removed under reduced pressure to give a pale yellow solid crude product. This crude product was purified by flash silica gel chromatography (eluant: CH_2Cl_2/CH_3OH 98:2) to obtain cholesteryl-3-carboxyamino-6-hexanol as a white solid (7.5 g, 80.1 % yield); TLC R_f 0.23 (CH_2Cl_2/CH_3OH 95:5).

Example 2

Synthesis of N,N,N',N'-tetraisopropyl-O-ethyl-phosphorodiamidite

Anhydrous ethanol (1.1 ml, 0.86 g, 18.7 mmol) in THF (4 ml) was added dropwise at 0 °C to a stirred solution of bis(diisopropylamino)chlorophosphine (5.0 g, 18.7 mmol) and triethylamine (3.92 ml, 2.84 g, 28.1 mmol) in THF (19 ml). After stirring for 1 hour at room temperature, the reaction mixture was filtered and the filtrate was concentrated to an oil. The oil was dissolved in 15 ml of anhydrous pentane and filtered. The solvent was removed under the reduced pressure. The mixture was again dissolved in 15 ml of anhydrous pentane and filtered to remove the remaining precipitates. The solvent was evaporated under the reduced pressure to give *O*-ethyl-phosphordiamidite as a colorless oil (4.83 g, 93.3% yield); ^{31}P NMR ($CDC1_3$) δ 137.79.

Example 3

Synthesis of N,N,N',N'-tetraisopropyl-O-isopropyl-phosphordiamidite

In a manner similar to that presented in Example 2, *O*-isopropyl-phosphordiamidite was obtained as a colorless oil (4.00 g, 73.4% yield) by using bis(diisopropylamino)chlorophosphine (5.0 g, 18.7 mmol), triethylamine (3.92 ml, 2.84 g, 28.1 mmol), anhydrous 2-propanol (1.43 ml, 1.13 g, 18.74 mmol) and THF (23 ml); ^{31}P NMR ($CDC1_3$) δ 126.95.

Example 4

Synthesis of N,N,N',N'-tetraisopropyl-O-(1-adamantyl-2-ethyl) -phosphordiamidite

In a manner similar to that presented in Example 2, *O*-(1-adamantyl-2-ethyl)-phosphordiamidite was obtained as a white solid (6.60 g, 86.3% yield) by using bis(diisopropylamino)chlorophosphine (5.0 g, 18.7 mmol), triethylamine (3.92 ml, 2.84 g, 28.1 mmol), 1-adamantaneethanol (3.38 g, 18.74 mmol) and THF (31 ml); ^{31}P NMR ($CDCl_3$) δ 136.68.

Example 5

Synthesis of N,N,N',N'-tetraisopropyl-O-(cholesteryl-3-carboxyamino-6-hexyl)-phosphordiamidite

In a manner similar to that presented in Example 2, *O*-(cholesteryl-3-carboxyamino-6-hexyl)- phosphordiamidite was obtained as a colorless sticky oil (4.38 g, 98.4 % yield) by using bis(diisopropylamino)chlorophosphine (1.56 g, 5.84 mmol), triethylamine (1.22 ml, 0.89 g, 8.76 mmol), cholesteryl-3-carboxyamino-6-hexanol (3.10 g, 5.84 mmol) and THF (3.6 ml); ^{31}P NMR ($CDC1_3$) δ 136.17.

Example 6

Synthesis of N,N,N',N'-tetraiospropyl-O-(1-hexadecyl)-phosphordiamidite

In a manner similar to that presented in Example 2, *O*-(1-hexadecyl)-phosphordiamidite was obtained as a colorless oil (7.82g, 88.1% yield) by using bis(diisopropylamino)chlorophosphine (5.0g, 18.74 mmol), triethylamine (3.92 ml, 2.84 g, 28.1 mmol), 1-hexadecanol (3.38 g, 18.74 mmol) and THF (29 ml); ^{31}P NMR ($CDC1_3$) δ 136.28.

Example 7

Synthesis of 5'-dimethoxytrityl-2'-deoxythymidine-N,N-diisopropyl-O-ethyl-phosphoramidite (5'-DMT-dT-O-ethyl-phosphoramidite)

O-ethyl-phosphordiamidite (1.02 g, 3.67 mmol) was added in one batch under nitrogen to a stirred suspension of 5'-DMT-T (1.0 g, 1. 84 mmol) in CH_2Cl_2 (5.0 ml). A solution of tetrazole (0.129 g, 1.84 mmol) in acetonitrile (4.7 ml) was added dropwise to the resulting mixture. After the mixture was stirred for 5 hours at room temperature, the solvent was removed at reduced pressure to give a foamy solid. The crude product was purified by flash column chromatography (eluant: CH_2Cl_2/EtOAc/Et_3N 70:20:10) and precipitated from hexane at -78 °C to give 5'-DMT-dT-*O*- ethyl-phosphoramidite as a white foam (0.62 g, 47.0% yield): TLC R_f 0.66 (CH_2Cl_2/EtOAc/NEt_3 45:45:10); ^{31}P NMR ($CDCl_3$) δ 157.73, 158.52.

Example 8

Synthesis of (5'-dimethoxytrityl-N^4-(4-tert-butylphenoxy)acetyl-2'-deoxycytidine-N,N-diisopropyl-O-ethyl-phosphoramidite (5'-DMT-dC^{tBA}-O-ethyl-phosphoramidite)

O-ethyl-phosphordiamidite (1.71 g, 4.64 mmol) in CH_2Cl_2 (9.7 ml) was added under nitrogen to a stirred pale yellow suspension of 5'-DMT-dC^{tBA} (2.25 g, 3.08 mmol) in CH_2Cl_2 (11.3 ml). A solution of tetrazole (0.216 g, 3.08 mmol) in acetonitrile (9.7 ml) was added dropwise to the resulting mixture. After the mixture was stirred for 5 hours at room temperature, the solvent was removed at reduced pressure to give a foamy solid. The crude product was purified by flash column chromatographly (eluant: CH_2Cl_2/EtOAc/Et_3N 70:20:10) and precipitation from hexane at -78°C to give 5'-DMT-dC^{tBA}-*O*-ethyl-phosphoramidite as a white foam (1.63 g, 58.5% yield): TLC R_f 0.80 (CH_2Cl_2/EtOAc/NEt_3 =45:45:10); ^{31}P NMR ($CDCl_3$) δ 158.20, 159.08.

Example 9

Synthesis of 5'-dimethoxytrityl-2'-deoxythymidine-N,N-diisopropyl-O-isopropyl-phosphoramidite (5'-DMT-dT-O-isopropyl-phosphoramidite)

O-isopropyl-phosphordiamidite (0.80 g, 2.8 mmol) in CH_2Cl_2 (2.0 ml) was added under nitrogen to a stirred suspension of 5'-DMT-T (1.0 g, 1.9 mmol) in CH_2Cl_2 (5.0 ml). A solution of tetrazole (0.129 g, 1.84 mmol) in acetonitrile (5.7 ml) was added to the resulting mixture. After the mixture was stirred for 5 hours at room temperature, the solvent was removed at reduced pressure to give a foamy solid. The crude product was purified by flash column

chromatography (eluant: CH_2Cl_2/EtOAc/Et_3N 70:20:10) and precipitated from hexane at -78 °C to give 5'-DMT-dT-*O*- isopropyl-phosphoramidite as a white foam (0.66 g, 48.7% yield): TLC R_f 0.70 (CH_2Cl_2/EtOAc/NEt_3 45:45:10); ^{31}P NMR ($CDCl_3$) δ 156.63, 157.14.

Example 10

Synthesis of 5'-dimethoxytriyl-N^4-(4-tert-butylphenoxy)acetyl-2'-deoxycytidine-N,N-diisopropyl-O-isopropyl-phosphoramidite (5'-DMT-dC^{tBA}-O-isopropyl-phosphoramidite)

O-isopropyl-phosphordiamidite (0.60 g, 2.06 mmol) in CH_2Cl_2 (2.0 ml) was added to a stirred pale yellow suspension of 5'-DMT-dC^{tBA} (1.0 g, 1.37 mmol) in CH_2Cl_2 (5.0 ml) under nitrogen. To the resulting mixture was added dropwise a solutioin of tetrazole (0.096 g, 1.37 mmol) in acetonitrile (4.3 ml). After the mixture was stirred for 5 hours at room temperature, the solvent was removed at reduced pressure to give a foamy solid. The crude product was purified by flash column chromatography (eluant:CH_2Cl_2/EtOAc/Et_3N 70:20:10) and precipitation from hexane at -78 °C to give 5'-DMT-dC^{tBA}-*O*-isopropyl-phosphoramidite as a white foam (0.84 g, 67.1% yield): TLC R_f 0.75 (CH_2Cl_2/EtOAc/NEt_3 45:45:10); ^{31}P NMR ($CDCl_3$) δ 157.24, 157.56.

Example 11

Synthesis of 5'-dimethoxytrityl-2'-deoxythymidine-N,N-diisopropyl-O-(1-adamantyl-2-ethyl)-phosphoramidite (5'-DMT-dT-O-1-adamantyl-2-ethyl)-phosphoramidite)

O-(1-adamantyl-2-ethyl)-phosphordiamidite (1.14 g, 2.76 mmol) in CH_2Cl_2 (5.7 ml) was added under nitrogen to a stirred suspension of 5'-DMT-dT (1.0 g, 1.8 mmol) in CH_2Cl_2 (5.0 ml). A solution of tetrazole (0.129 g, 1.84 mmol) in acetonitrile (5.7 ml) was added dropwise to the resulting mixture. After the mixture was stirred for 5 hours at room temperature, the solvent was removed at reduced pressure to give a foamy solid. The crude product was purified by flash column chromatography (eluant: CH_2Cl_2/EtOAc/Et_3N 70:20:10) and precipitated from hexane at -78°C to give 5'-DMT-dT-*O*-(1-adamantyl-2-ethyl)-phosphoramidite as a white foam (0. 86 g, 54.7% yield): TLC R_f 0.65 (CH_2Cl_2/EtOAc/NEt_3 45:45:10); ^{31}P NMR ($CDCl_3$) δ 157.93, 158.31.

Example 12

Synthesis of 5'-dimethoxytrityl-N^4-(4-tert-butylphenoxy)acetyl-2'-deoxycytidine-N,N-diisopropyl-O-(1-adamantyl-2-ethyl)-phosphoramidite (5'-DMT- dC^{tBA}-O-(1-adamantyl-2-ethyl)-phosphoramidite)

O-(1-adamantyl-2-ethyl)-phosphordiamidite (0.85 g, 2.06 mmol) in CH_2Cl_2 (4.3 ml) was added under nitrogen to a stirred pale yellow suspension of 5'-DMT-dC^{tBA} (1.0 g, 1.4 mmol) in CH_2Cl_2 (5.0 ml). A solution of tetrazole (0.096 g, 1.37 mmol) in acetonitrile (4.3 ml) was added dropwise to the resulting mixture. After the mixture was stirred for 5 hours at room temperature, the solvent was removed at reduced pressure to give a foamy solid. The crude product was purified by flash column chromatography (eluant: CH_2Cl_2/EtOAc/Et_3N 70:20:10) and precipitated from hexane at -78°C to give 5'-DMT-dC^{tBA}-*O*-(1-adamantyl-2-ethyl)-phosphoramidite as a white foam (0.83 g, 58.6%, yield): TLC R_f 0.63 (CH_2Cl_2/EtOAc/NEt_3 45:45:10); ^{31}P NMR ($CDCl_3$) δ 158.35, 158.67.

Example 13

Synthesis of 5'dimethoxytrityl-2'-deoxythymidine-N,N-diisopropyl-O-(cholesteryl-carboxyamino-6-hexyl)-phosphoramidite (5'-DMT-dT-O-(cholesteryl-3-carboxyamino-6-hexyl)-phosphoramidite)

O-(cholesteryl-3-carboxyamino-6-hexyl)-phosphordiamidite (2.1 g, 2.76 mmol) in CH_2Cl_2 (11.9 ml) was added under nitrogen to a stirred suspension of 5'-DMT-T (1.0 g, 1.84 mmol) in CH_2Cl_2 (5.0 ml). A solution of tetrazole (0.129 g, 1.84 mmol) in acetonitrile (7.5 ml) was added dropwise to the resulting mixture. After the mixture was stirred for 5 hours at room temperature, the solvent was removed at reduced pressure to give a white foamy solid. The crude product was purified by flash column chromatography (eluant: CH_2Cl_2/EtOAc/Et_3N 70:20:10) to give 5'-DMT-dT-*O*(cholesteryl-3-carboxyamino-6-hexyl)-phosphoramidite as a white foam (2.11 g, 95.2% yield): TLC R_f 0.60 (CH_2Cl_2/EtOAc/NEt_3 45:45:10); ^{31}P NMR ($CDCl_3$) δ 158.60, 158.82.

Example 14

Synthesis of 5'-dimethoxytrityl-N^4-(4-tert-butylphenoxy)acetyl-2'-deoxycytidine-N,N-diisopropyl-O-(cholesteryl-3-carboxyamino-6-hexyl)-phosphoramidite (5'-DMT-dC^{BA}-O-(cholesteryl-3-carboxyamino-6-hexyl)-phosphoramidite)

O-(cholesteryl-3-carboxyamino-6-hexyl)-phosphordiamidite (2.35 g, 3.09 mmol) in CH_2Cl_2 (13.1 ml) was added under nitrogen to a stirred pale yellow suspension of 5'-DMT-dC^{bz} (1.5 g, 2.06 mmol) in CH_2Cl_2 (7.5 ml). A solution of tetrazole (0.144 g, 2.06 mmol) in acetonitrile (8.4 ml) was added dropwise to the resulting mixture. After the mixture was stirred for 5 hours at room temperature, the solvent was removed at reduced pressure to give a foamy solid. The crude product was purified by flash column chromatography (eluant: CH_2Cl_2/EtOAc/Et_3N 70:20:10) to give 5'-DMT-dC^{BA}-*O*-(cholesteryl-3-carboxyamino-6-hexyl)-phosphoramidite as a white foam (2.58 g, 90.4% yield): TLC R_f 0.63 (CH_2Cl_2/EtOAc/NEt_3 45:45:10); ^{31}P NMR ($CDCl_3$) δ 158.93, 159.25.

Example 15

Synthesis of 5'-dimethoxytrityl-2'-deoxythymidine-N,N-diisopropyl-O-(1-hexadecyl)-phosphoramidite (5'-DMT-dT-O-(1-hexadecyl)-phosphoramidite)

O-(1-hexadecyl)-phosphordiamidite (1.31 g, 2.76 mmol) in CH_2Cl_2 (5.7 ml) was added under nitrogen to a stirred suspension of 5'-DMT-T (1.0 g, 1.84 mmol) in CH_2Cl_2 (5.0 ml). A solution of tetrazole (0.129 g, 1.84 mmol) in acetonitrile (5.7 ml) was added dropwise to the resulting mixture. After the mixture was stirred for 5 hours at room temperature, the solvent was removed at reduced pressure to give a foamy solid. The crude product was purified by flash column chromatography (eluant: CH_2Cl_2/EtOAc/Et_3N 70:20:10) to give 5'-DMT-dT-*O*-(1-hexadecyl)-phos-phoramidite as a colorless sticky oil (1.65 g, 98.0% yield): TLC R_f 0.63 (CH_2Cl_2/EtOAc/NEt_3 45:45:10); ^{31}P NMR ($CDCl_3$) δ 159.00, 158.32.

Example 16

Synthesis of 5'-dimethoxytrityl-N⁴-(4 -tert-butylphenoxy)acetyl-2'-deoxycytidine-N,N-diisopropyl-O-(1-hexadecyl)-phosphoramidite (5'-DMT- dC^{tBA}-O-(1-hexadecyl)-phosphoramidite)

O-(1-hexadecyl)-phosphordiamidite (0.97 g, 2.06 mmol) in CH_2Cl_2 (4.3 ml) under nitrogen was added under nitrogen to a stirred pale yellow suspension of 5'-DMT-dC^{tBA} (1.0 g, 1.37 mmol) in CH_2Cl_2 (5.0 ml). A solution of tetrazole (0.096 g, 1.37 mmol) in acetonitrile (4.3 ml) was added dropwise to the resulting mixture. After the mixture was stirred for 5 hours at room temperature, the solvent was removed at reduced pressure to give a foamy solid. The crude product was purified by flash column chromatography (eluant: CH_2Cl_2/EtOAc/Et_3N 70:20:10) to give 5'-DMT-dC^{tBA}-*O*-(1-hexadecyl)-phosphoramidite as a pale yellow sticky oil (1.41 g, 93.5% yield): TLC R_f 0.62 (CH_2Cl_2/EtOAc/NEt_3 45:45:10); ^{31}P NMR ($CDCl_3$) δ 158.71, 159.47.

Example 17

Synthesis of O-alkyl-phosphoramidite

The syntheses of a number of *O*-alkyl-phosphoramidites have been reported, which include methyl, ethyl, trifluoroethyl, isopropyl and neopentyl derivatives. Koziolkiewicz and Wilk in *Protocols for Oligonucleotides and Analogs, supra,* pp. 207-224, and references cited therein. In a similar way, the O-alkyl-phosphordiamidites were prepared by the reaction of bis(diisopropylamino)chlorophosphine with the alcohols. In purification of the O-alkylphosphoramidites, vacuum distillation at rather high temperature was replaced by the repeated filtration of the corresponding pentane solution to avoid the possible decomposition of the product. The phosphitylation of the nucleosides by the resulting *O*-alkyl-phosphordiamidites gave the *O*-alkyl-phosphoramidites in good to excellent yield. The synthesis of the *O*-alkyl-phosphoramidites is depicted in Figure 1.

Example 18

Synthesis of S-triester-phosphorothioate and O-triester-phosphotriester oligonucleotides

The S-triester-phosphorothioate and *O*-triester-phosphodiester oligonucleotides were synthesized using the phosphoramidites of Example 17 protected by the base labile *tert*-butylphenoxyacetyl (tBA) group on the exocyclic amine (dA, dC, and dG). The increased liability of tBA group over the standard benzoyl and isobutyryl protection permitted use of milder condition for deprotection of the bases and release from the solid support with ammonia hydroxide (room temperature, 2 hours), which was of crucial importance in the synthesis of the *O*-ethyl containing oligonucleotides. Oligonucleotides were purified by reverse-phase HPLC and/or PAGE. The ^{31}P NMR spectrum of the S-triester-phosphorothioate compound no. 1.1 showed that the corresponding peaks for S-triester-phosphorothioate and S-phosphorothioate internucleotide linkages appear at 62 and 53 ppm respectively. The ratio between S-triester and phosphorothioate was 19.8 :100.0 (The calculated value is 20:100). The content of O-phosphodiester linkages was shown to be less than 2.5 percent.

Example 19

Synthesis and purification of oligonucleotides

All of unmodified or triester containing phosphorothioate and phosphodiester oligonucleotides were synthesized on a 1 μmol scale following the standard protocol by using an automated synthesizer (Millipore 8909 Expedite, Bedford, MA). The modified phosphoramidites, 5'-DMT-dT-*O*-ethyl-phosphoramidite, 5'-DMT-dC^{tBA}-*O*-ethyl-phosphoramidite, 5'-DMT-dT-*O*-iso-propyl-phosphoramidite, and 5'-DMT-dC^{tBA}-*O*-isopropyl-phosphoramidite were dissolved in dry acetonitrile at a concentration of 50 mg/ml, and the others, 5'-DMT-dT-*O*-(1-adamantyl-2-ethyl)- phosphoramidite, 5'-DMT-dC^{tBA}-adamantyl-2-ethyl)-phosphoramidite, 5'-DMT-dT-*O*-(cholesteryl-3-carboxyamino-6-hexyl)-phosphoramtidite, 5'-DMT-dC^{tBA}-*O*-(cholesteryl-3-carboxyamino-6-hexyl)-phosphoramidite, 5'-DMT-dT-*O*-(1-hexadecyl)-phosphoramidite, and 5'-DMT-dC^{tBA}-*O*-(1-hexadecyl)-phosphoramidite were dissolved in dichloromethane and diluted with acetonitrile to acetonitrile-dichloromethane (1:1) at a final concentration of 50 mg/ml. For phosphorothioate oligonucleotides, the iodine oxidation step was replaced by sulfurization with 3H-1,2-benzodithiol-3-one-1,1-dioxide (Beaucage reagent). Iyer et al., *J. Org. Chem* 55, 4693 (1990). Two-hour treatment with ammonium hydroxide at room temperature was carried out to

cleave the oligomer from the support and to deprotect nucleoside bases. Oligonucleotides were purified by reverse-phase HPLC and/or PAGE, and desalted by using C-18 SEP-PAK cartridges.

Fluorescein labeling of oligonucleotides

Fluorescein was conjugated to the 5' end of the oligonucleotides by either an automated DNA synthesizer or by a manual procedure using a FLUORESCEIN-ON™ phosphoramidite. The efficiency of fluorescein labeling was determined by using a spectrofluorometer (excitation 488 nm, emission 520 nm).

Example 20

Exonuclease resistance of triester containing oligonucleotides

Sensitivity to 3'-exonuclease degradation was measured by digestion with T4 DNA polymerase and/or DNA polymerase I. The oligonucleotides tested are listed in Table 1.

Table 1

No.	Sequence[a]	Modification[b]
1.1	CTC TCG CAC CCA TCT CTC TCC TTC T	--
1.4[c]	CTC TCG CAC CCA TCT CTC TCC TTC T	R_1
1.6	CTC TCG CAC CCA TCT CTC TCC TTC T	R_2
1.8	CTC TCG CAC CCA TCT CTC TCC TTC T	R_3
1.10	CTC TCG CAC CCA TCT CTC TCC TTC T	R_3
1.12	CTC TCG CAC CCA TCT CTC TCC TTC T	R_3
1.15	CTC TCG CAC CCA TCT CTC TCC TTC T	R_4
1.17	CTC TCG CAC CCA TCT CTC TCC TTC T	R_5

[a] All compounds are oligodeoxyribonucleotides and are presented in the 5' to 3' direction, left to right; highlighted nucleotides have S-triester linkages on the 3' side, except as otherwise indicated.

[b] R_1=ethyl; R_2=isopropyl; R_3=1-adamantyl-2-ethyl; R_4=Cholesteryl-3-carboxyamino-6-hexyl; R_5=1-hexadecyl; where one R group is listed, that R group is in each S-triester or O-triester linkage; where there are two or more R groups listed, each R group corresponds to one S-triester or O-triester linkage, with the first listed R group being in the 5'-most S-triester or O-triester linkage and so on so that the last R group listed corresponds to the 3'-most S-triester or O-triester linkage.

[c] Highlighted nucleotides have O-triester linkages on the 3' side.

To study the resistance f T4 DNA polymerase, 5'-^{32}P-labeled oligonucleotides (45 pmole) were dissolved in 30 µl of buffer (50 mM Tris, pH 8.0, $MgCl_2$, 5 mM DTT, 0.05% BSA) and incubated with T4 DNA polymerase (7.5 units) at 37°C . Aliquots (5 µl), inhibited by 6 µl of the stop solution (95% formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), were removed at 0 and 60 minutes, analyzed by PAGE (20% polyacrylamide containing 8.3 M urea), and followed by autoradiography. The results are shown in Fig. 2.

To study the resistance of DNA polymerase I, 5'- ^{32}P labeled oligonucleotides (30 pmole) were dissolved in 20 µl of buffer (50 mM Tris, pH 8.0, $MgCl_2$, 5 mM DTT, 0.05% BSA) and incubated with DNA polymerase I (5.0 units) at 37 °C. Aliquots (5 µl), inhibited by 6 µl of the stop solution (95% formamide, 10 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), were removed at 0, 60, 120, 180 and 240 minutes, analyzed by PAGE (20% polyacrylamide containing 8.3 M urea), and followed by autoradiography. Typical results are shown in Fig. 3.

The results clearly indicate that the modified S-triester-phosphorothioate is much more stable than the unnodified S-phosphorothioate. Fig. 4 The exonuclease resistance of other modified S-triester-phosphorothioates and O-triester-phosphodiesters oligonucleotide was also studied by using T4 polymerase (Fig. 2). It is seen that not only the modified S-triester-phosphorothioate but also the O-triester-phosphodiester oligonucleotides have increased exonuclease resistance. This is important because the S-triester-phosphorothioate may convert to O-triester-phosphodiester in cells (Wyrzykiewicz and Cole, *Nucleic Acids Res.* **22**, 2667 (1994)), and the increased persistence of O-triester-phosphodiester would provide a longer duration of action *in vivo*.

Example 21

Melting Temperatures (T_m)

Melting temperatures were determined for the duplexes of unmodified and modified oligonucleotides with the complementary DNA. Each oligonucleotide (0.2 A_{260} Units) and its complementary DNA was annealed in 1 ml buffer (10 mM Na_2HPO_4, pH 7.4, 0.1 M NaCl) by heating to 80 °C and then cooling down to 40 °C at a rate of 2 °C/minute. The mixture was then reheated to 80 °C at a rate of 1° C/minute and the A_{260} was continuously recorded. Melting profiles were obtained for oligonucleotides.

The results are presented in Table 2. Changes in affinity can be attributed directly to the modification. Modification with triester linkage(s) at the 3' end and/or the 5' end of the S-phosphorothioate reduces the total negative charge of the oligonucleotide, which would be expected to enhance hybridization. As Table 2 shows, almost all of the modified S-triester-phosphorothioates have increased T_m compared to the unmodified phosphorothioate (compound no. 1.0). The only exception is a chimeric oligomer (compound no. 1.2) modified at both of the 3' and 5' ends with four ethyl triester linkages. The decreased T_m indicates that the modification has caused changes in the geometry of the internucleotide bond. It is also shown that the melting temperatures of O-triester-phosphodiesters are about 10 degrees higher than that of the corresponding S-triester-phosphorothioates, which is consistent with the results obtained from the unmodified oligonucleotides. Uhlmann and Peyman, *supra*; and *Methods in Molecular Biology 20: Protocols for Oligonucleotides and Analogs, supra*.

Table 2

Melting Temperatures of Various Modifications of SEQ ID NO 1

No.	Sequence[a]	Modification[b]	T_m(°C)
1.0	CTC TCG CAC CCA TCT CTC TCC TTC T	--	52.2
1.1	CTC TCG CAC CCA TCT CTC TC<u>C TTC</u> T	R_1	56.8

Table 2 (c nt.)

No.	Sequence[a]	Modification[b]	T_m(°C)
1.2	CTC TCG CAC CCA TCT CTC TCC TTC T	R_1	51.4
1.3	CTC TCG CAC CCA TCT CTC TCC TTC T	R_1	55.6
1.4[c]	CTC TCG CAC CCA TCT CTC TCC TTC T	R_1	65.8
1.5[c]	CTC TCG CAC CCA TCT CTC TCC TTC T	R_1	65.4
1.6	CTC TCG CAC CCA TCT CTC TCC TTC T	R_2	55.5
1.7	CTC TCG CAC CCA TCT CTC TCC TTC T	R_2	54.2
1.8	CTC TCG CAC CCA TCT CTC TCC TTC T	R_3	55.2
1.9	CTC TCG CAC CCA TCT CTC TCC TTC T	R_3	54.1
1.10	CTC TCG CAC CCA TCT CTC TCC TTC T	R_3	55.9
1.11	CTC TCG CAC CCA TCT CTC TCC TTC T	R_3	57.0
1.12	CTC TCG CAC CCA TCT CTC TCC TTC T	R_3	56.8
1.13	CTC TCG CAC CCA TCT CTC TCC TTC T	R_3	55.8
1.14	CTC TCG CAC CCA TCT CTC TCC TTC T	R_3	55.0
1.15	CTC TCG CAC CCA TCT CTC TCC TTC T	R_4	57.5
1.16	CTC TCG CAC CCA TCT CTC TCC TTC T	R_4	57.2

Table 2 (cont.)

No.	Sequence[a]	Modification[b]	T_m(°C)
1.17	CTC TCG CAC CCA TCT CTC TCC TTC T	R_5	56.1
1.18	CTC TCG CAC CCA TCT CTC TCC TTC T	R_5	56.8
1.19	CTC TCG CAC CCA TCT CTC TCC TTC T	R_5	54.4
1.20	CTC TCG CAC CCA TCT CTC TCC TTC T	R_5	54.1
1.21	CTC TCG CAC CCA TCT CTC TCC TTC T	R_1, R_4	53.6
1.22	CTC TCG CAC CCA TCT CTC TCC TTC T	R_1, R_4	57.1
1.23	CTC TCG CAC CCA TCT CTC TCC TTC T	R_1, R_1, R_4	55.2
1.24	CTC TCG CAC CCA TCT CTC TCC TTC T	R_1, R_1, R_4	55.2
1.25	CTC TCG CAC CCA TCT CTC TCC TTC T	R_1, R_1, R_4	56.4
1.26	CTC TCG CAC CCA TCT CTC TCC TTC T	R_1, R_1, R_4	53.6
1.27[c]	CTC TCG CAC CCA TCT CTC TCC TTC T	R_1, R_4	65.7
1.28[c]	CTC TCG CAC CCA TCT CTC TCC TTC T	R_1, R_4	66.8
1.29[c]	CTC TCG CAC CCA TCT CTC TCC TTC T	R_5	66.0
1.30	CTC TCG CAC CCA TCT CTC TCC TTC T	R_1	56.3
1.31	CTC TCG CAC CCA TCT CTC TCC TTC T	R_5	54.5
1.32	CTC TCG CAC CCA TCT CTC TCC TTC T	R_5, R_1	52.5

[a] All compounds are oligodeoxyribonucleotides and are presented in the 5' to 3' direction, left to right; highlighted nucleotides have an S-triester linkage at the 3' side, except as indicated.

[b] R_1=ethyl; R_2=isopropyl; R_3=1-adamantyl-2-ethyl; R_4=Cholesteryl-3-carboxyamino-6-hexyl; R_5=1-hexadecyl; where one R group is listed, that R group is in each S-triester or O-triester linkage; where there are two or more R groups listed, each R group corresponds to one S-triester or O-triester linkage, with the first listed R group being in the 5'-most S-triester or O-triester linkage and so on so that the last R group listed corresponds to the 3'-most S-triester or O-triester linkage.

[c] Highlighted nucleotides have an O-triester linkage on the 3' side.

Example 22

Cellular Uptake

Cell culture: Human T cell and leukemia cell line H9 were used in the study. They were cultured in RPMI media supplemented with 10% fetal bovine serum (heat inactivated to 56 °C for 30 minutes to inactivate the nucleases), 2 mM glutamine, 100 µl streptomycin, 100 U/mL penicillin and 6 x 10^{-5} M of 2-mercaptoethanol in an air incubator (37° C, humidified by 5% CO_2-95% O_2).

Cell uptake: The concentrations of the fluorescein labeled and unlabelled oligonucleotides in the samples were measured by a spectrofluorometer and UV spectroscopy, and adjusted to be same by adding the corresponding unlabelled oligonucleotides. Oligonucleotide complexes (0.2 OD/100 µl) were added to the cells (5 x 10^6 cells/ml, 0.5 ml) and set to culture. After 4 hours of culture, aliquots of cell culture mixtures were removed, washed, and resuspended in Hank's balanced salt solution (HBSS) supplemented with 0.1 % BSA and 0.1 % sodium azide. Propidium iodide (final concentration 10 µl/ml) was used to distinguish viable cells from dead cells. Flow cytometric data on 5,000 viable cell was acquired in list mode on Epics XL (Coulter, Hialeah, FL), and data were analyzed by Epics XL (version 1.5 software) after gating on living cells by forward scatter versus side scatter and propidium iodide staining.

The results are presented in Table 3. It is seen that the S-triester-phosphorothioates modified with a large lipophilic group (*e.g.*, 1-adamantyl-2-ethyl, cholesteryl-3-carboxyamino-6-hexyl and 1-hexadecyl) have enhanced cellular uptake.

Table 3

Cellular Uptake of SEQ ID No. 1

No.	Sequence[a]	Modification[b]	Cell Uptake[c]
1.0	CTC TCG CAC CCA TCT CTC TCC TTC T	--	7.4
1.1	CTC TCG CAC CCA TCT CTC TC**C TTC** T	R_1	11.4
1.3	**C**TC TCG CAC CCA TCT CTC TC**C TTC** T	R_1	5.7
1.6	CTC TCG CAC CCA TCT CTC TC**C TTC** T	R_2	7.9
1.8	CTC TCG CAC CCA TCT CTC TCC T**TC** T	R_3	14.1
1.12	CTC TCG CAC CCA TCT CTC TCC TT**C** T	R_3	11.6
1.15	CTC TCG CAC CCA TCT CTC TCC TT**C** T	R_4	19.9
1.17	CTC TCG CAC CCA TCT CTC TCC TT**C** T	R_5	19.8

[a] All compounds are oligodeoxyribonucleotides and are presented in the 5' to 3' direction, left to right; highlighted nucleotides have an S-triester linkage at the 3' side.

[b] Each of the S-triester linkages have the indicated R group, where R_1=ethyl, R_2=isopropyl, R_3=1-adamantyl-2-ethyl, R_4=Cholesteryl-3-carboxyamino-6-hexyl, and R_5=1-hexadecyl.

[c] Mean FITC fluorescence.

Example 23

Inhibition of CMV Gene Expression in Cell Culture

A series of modified CMV oligonucleotides were synthesized according to the invention. These oligonucleotides are depicted in Table 4.

Table 4

Modification of SEQ ID NO 2 Used to Inhibit CMV Gene Expression

No.	Sequence[a]	Modification[b]
2.0	TGG GGC TTA CCT TGC GAA CA	R_3
2.1	TGG GGC TTA CCT TGC GAA CA	R_1, R_3
2.2[c]	TGG GGC TTA CCT TGC GAA CA	R_3
2.3[c]	TGG GGC TTA CCT TGC GAA CA	R_1, R_3
2.4	TGG GGC TTA CCT TGC GAA CA	R_1
2.5	TGG GGC TTA CCT TGC GAA CA	R_2
2.6	TGG GGC TTA CCT TGC GAA CA	R_1, R_2
2.7	TGG GGC TTA CCT TGC GAA CA	R_4
2.8	TGG GGC TTA CCT TGC GAA CA	R_1, R_4
2.9	TGG GGC TTA CCT TGC GAA CA	R_1
2.10[c]	TGG GGC TTA CCT TGC GAA CA	R_1, R_4
2.11[c]	TGG GGC TTA CCT TGC GAA CA	R_4
2.12[c]	TGG GGC TTA CCT TGC GAA CA	R_1
2.13[c]	TGG GGC TTA CCT TGC GAA CA	R_1

[a] All compounds are oligodeoxyribonucleotides and are presented in the 5' to 3' direction, left to right; highlighted nucleotides have S-triester linkages on the 3' side, except as otherwise indicated.

[b] R_1=ethyl; R_2=1-adamantyl-2-ethyl; R_3=Cholesteryl-3-carboxyamino-6-hexyl; R_4=1-hexadecyl; where one R group is listed, that R group is in each S-triester or O-triester linkage; where there are two or more R groups listed, each R group corresponds to one S-triester or O-triester linkage, with the first listed R group being in the 5'-most S-triester or O-triester linkage and so on so that the last R group listed corresponds to the 3'-most S-triester or O-triester linkage.

[c] Highlighted nucleotides have O-triester linkages on the 3' side.

The sequence depicted in Table 4, 5'-TGGGGCTTACCTTGCGAACA-3' (UL36 ANTI), is complementary to the intron-exon boundary of UL36/37 and to the control sense oligonucleotide 5'-TGTTCGCAAGGTAAGCCCCA-3', which is homologous to the UL36 mRNA. These oligonucleotides correspond to HCMV genomic corrdinates 49,565 to 49,584.

Smith and Pari, *J. Virology* 69, 1925 (1995). In addition, two other compounds were used as controls in the experiments described below: 5'-TCTGGGTAATTACACGCAAGC-3', an unrelated nonspecific control oligonucleotide, and 5'-ACAAGCGTTCCATTCGGGGT-3', a nonsense control oligonucleotide that is the same nucleotide sequence as UL36 ANTI, except in reverse order.

Human foreskin fibroblasts (HFF) were plated at a density of 5,000 cells per well in a 96-well microtiter plate (Falcon, Franklin Lakes, N.J.) for 24 hr prior to treatment. Cells were pretreated with antisense oligonucleotides at the various indicated concentrations in CCM5 medium (Hyclone, Logan, Utah) for up to 15 hr. The growth medium was then removed, and the cells were washed three times with Dulbecco's phosphate-buffered saline to remove any residual oligonucleotide. The cells were incubated with HCMV strain AD169 (ATCC VR-538) at a multiplicity of infection (MOI) of 0.1 for 1 hr at 37°C. Cells were washed again and refed in fresh growth medium containing serial dilutions of the antisense oligonucleotide at the same concentrations used in the preincubation. At 5 to 6 days postinfection (p.i.), cells were fixed (100% ethanol) and reacted with a primary antibody specific for the HCMV UL44 gene product (Advanced Biotechnologies, Rivers Park, IL). The cells were then reacted with anti-mouse immunoglobulin G conjugated to horseradish peroxidase-labeled (kerkgaard and Perry, Gaithersburg, MD) secondary antibody and developed with a TMB substrate, and the optical density at 450 nm was determined by using a plate reader (Ceres 900, Biotek, Winooski, VT).

These ELISA experiments showed that some of these modified oligonucleotides almost completely inhibit HCMV DNA replication when used at concentrations as low as 0.08 μM. The results are presented in Table 5.

Table 5

Thiono Triester Oligonucleotide Phosphorothioate Inhibition of HCMV DNA Replication

No.	Sequence	Modification	Activity	
			0.08 μM	0.4 μM
2.0	TGG GGC TTA CCT TGC GAA CA	R_3	+	+
2.1	TGG GGC TTA CCT TGC GAA CA	R_1, R_3	+	+
2.2[c]	TGG GGC TTA CCT TGC GAA CA	R_3	-	n.t.

Table 5 (cont.)

No.	Sequence	Modificati n	Activity	
			0.08 μM	0.4 μM
2.3[c]	TGG GGC TTA CCT TGC GAA CA	R_1, R_3	-	n.t.
2.4	TGG GGC TTA CCT TGC GAA CA	R_1	-	n.t.
2.5	TGG GGC TTA CCT TGC GAA CA	R_2	-	-
2.6	TGG GGC TTA CCT TGC GAA CA	R_1, R_2	-	-
2.7	TGG GGC TTA CCT TGC GAA CA	R_4	-	+
2.8	TGG GGC TTA CCT TGC GAA CA	R_1, R_4	-	+
2.9	TGG GGC TTA CCT TGC GAA CA	R_1	-	+
2.10[c]	TGG GGC TTA CCT TGC GAA CA	R_1, R_4	-	-
2.11[c]	TGG GGC TTA CCT TGC GAA CA	R_4	-	-
2.12[c]	TGG GGC TTA CCT TGC GAA CA	R_1	-	n.t.
2.13[c]	TGG GGC TTA CCT TGC GAA CA	R_1	n.t.	n.t.

[a] All compounds are oligodeoxyribonucleotides and are presented in the 5' to 3' direction, left to right; highlighted nucleotides have S-triester linkages on the 3' side, except as otherwise indicated.

[b] R_1=ethyl; R_2=1-adamantyl-2-ethyl; R_3=Cholesteryl-3-carboxyamino-6-hexyl; R_4=1-hexadecyl; where one R group is listed, that R group is in each S-triester or O-triester linkage; where there are two or more R groups listed, each R group corresponds to one S-triester or O-triester linkage, with the first listed R group being in the 5'-most S-triester or O-triester linkage and so on so that the last R group listed corresponds to the 3'-most S-triester or O-triester linkage.

[c] Highlighted nucleotides have O-triester linkages on the 3' side.

n.t. = not tested

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Hybridon, Inc.

(ii) TITLE OF INVENTION: Thi n Triester Modified Antisense Oligodeoxynucleotide Phosphorothioates

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Banner & Allegretti, Ltd.
(B) STREET: 10 S. Wacker Drive Suite 3000
(C) CITY: Chicago
(D) STATE: Illinois
(E) COUNTRY: U.S.A.
(F) ZIP: 60606

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Greenfield, Michael S.
(B) REGISTRATION NUMBER: 37,142
(C) REFERENCE/DOCKET NUMBER: 94,1033

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (312)715-1000
(B) TELEFAX: (312)715-1234

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCTCGCACC CATCTCTCTC CTTCT 21

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGGGGCTTAC CTTGCGAACA 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGTTCGCAAG GTAAGCCCCA 20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCTGGGTAAT TACACGCAAG C 21

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

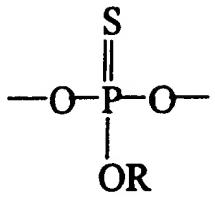
(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

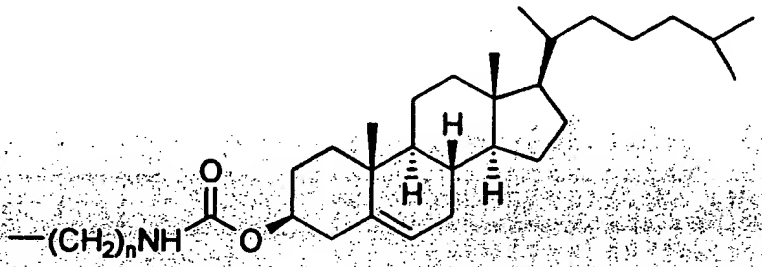
ACAAGCGTTC CATTCGGGGT 20

We claim:

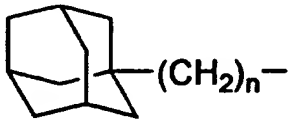
1. An oligonucleotide comprising from 1 to about 10 thiono-triester phosphorothioate internucleotide linkages having the structure:



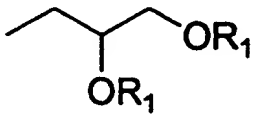
wherein the R group of each thiono-triester phosphorothioate internucleotide linkage is independently a C_2-C_{22} linear or branched chain alkyl group, a cholesteryl derivative having the structure:



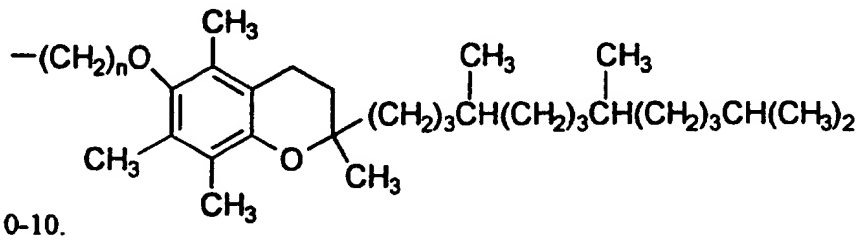
where n = 2-12, an adamantyl derivative having the strucutre:



where n = 2-12, a 1,2-di-*O*-alkyl-*rac*-3-glyceryl derivative having the structure:



where R_1 is C_nH_{2n+1} and n = 2-22, or a DL α-tocopherol derivative having the structure:



where n = 0-10.

2. An oligonucleotide according to claim 1 having from 2 to 100 nucleotides.

3. An oligonucleotide according to claim 1 having from 13 to 50 nucleotides.

4. An oligonucleotide according to claim 1 having from 20 to 35 nucleotides.

5. An oligonucleotide according to claim 3 wherein R is chosen from the group consisting of 1-adamantyl-2-ethyl, 1-hexadecyl, and cholesteryl-3-carboxyamino-6-hexyl.

6. An oligonucleotide according to claim 4 wherein R is chosen from the group consisting of 1-adamantyl-2-ethyl, 1-hexadecyl, and cholesteryl-3-carboxyamino-6-hexyl.

7. An oligonucleotide according to claim 4 having the formula 5'-TGGGGCTTACCTTGCGAACA-3' (SEQ ID NO 2).

8. A composition for inhibiting the replication of an infectious agent or gene expression of an infectious agent in a cell comprising an oligonucleotide according to claim 1 and a pharmaceutically acceptable carrier, wherein the oligonucleotide is complementary to a region of a nucleic acid essential to the replication of the infectious agent or expression of the gene.

9. A composition for inhibiting the replication of an infectious agent or gene expression of an infectious agent in a cell comprising an oligonucleotide according to claim 4 and a pharmaceutically acceptable carrier, wherein the oligonucleotide is complementary to a region of a nucleic acid essential to the replication of the infectious agent or expression of the gene.

10. A composition for inhibiting the replication of an infectious agent or gene expression of an infectious agent in a cell comprising an oligonucleotide according to claim 6 and a pharmaceutically acceptable carrier, wherein the oligonucleotide is complementary to a region of a nucleic acid essential to the replication of the infectious agent or expression of the gene.

11. A composition according to claim 8 wherein the infectious agent is CMV.

12. A composition for inhibiting the expression of a nucleic acid whose expression product causes or is involved in a diseased state comprising an oligonucleotide according to claim 1 and

a pharmaceutically acceptable carrier, wherein the oligonucleotide is complementary to a region of the nucleic acid.

13. A composition for inhibiting the expression of a nucleic acid whose expression product causes a diseased state comprising and oligonucleotid according to claim 4 and a pharmaceutically acceptable carrier, wherein the oligonucleotide is complementary to a region of the nucleic acid.

14. A composition for inhibiting the expression of a nucleic acid whose expression product causes a diseased state comprising and oligonucleotide according to claim 6 and a pharmaceutically acceptable carrier, wherein the oligonucleotide is complementary to a region of the nucleic acid.

15. A method of inhibiting the replication of an infectious agent or gene expression of an infectious agent in a cell comprising contacting the cell with an effective amount of an oligonucleotide according to claim 1, wherein the oligonucleotide is complementary to a region of a nucleic acid essential to the replication of the infectious agent or expression of the gene.

16. A method of inhibiting the replication of an infectious agent or gene expression of an infectious agent in a cell comprising contacting the cell with an effective amount of an oligonucleotide according to claim 4, wherein the oligonucleotide is complementary to a region of a nucleic acid essential to the replication of the infectious agent or expression of the gene.

17. A method of inhibiting the replication of an infectious agent or gene expression of an infectious agent in a cell comprising contacting the cell with an effective amount of an oligonucleotide according to any one of claims 6, wherein the oligonucleotide is complementary to a region of a nucleic acid essential to the replication of the infectious agent or expression of the gene.

18. A method of inhibiting the expression of a nucleic acid whose expression product results in a diseased state comprising contacting the nucleic acid with an effective amount of an oligonucleotide according to claim 1, wherein the oligonucleotide is complementary to a region of the nucleic acid.

19. A method of inhibiting the replication of an infectious agent or gene expressi n of an infectious agent in a cell comprising contacting the cell with an effective amount of an oligonucleotide according to claim 4, wherein the oligonucleotide is complementary to a region of the nucleic acid.

5

20. A method of inhibiting the replication of an infectious agent or gene expression of an infectious agent in a cell comprising contacting the cell with an effective amount of an oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of the nucleic acid.

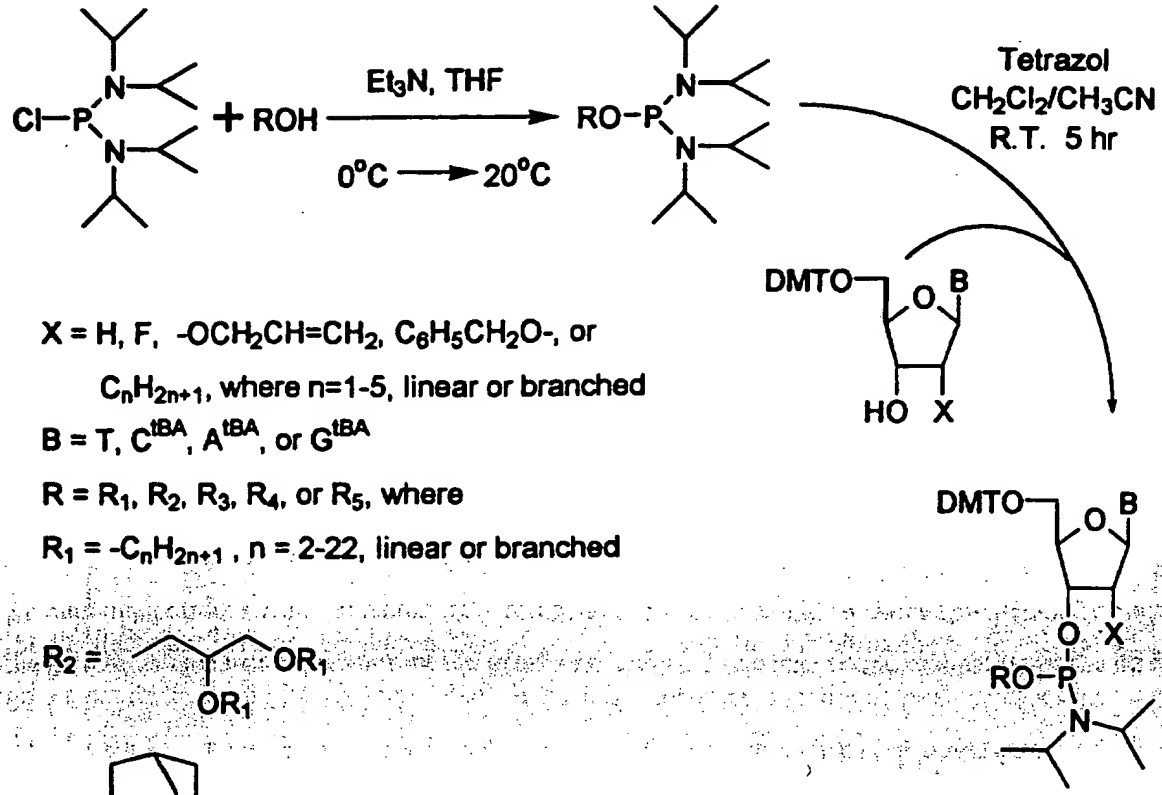

Et3N, THF
0°C → 20°C
Tetrazol
CH2Cl2/CH3CN
R.T. 5 hr
DMTO
HO X
X = H, F, -OCH2CH=CH2, C6H5CH2O-, or
CnH2n+1, where n=1-5, linear or branched
B = T, C^tBA, A^tBA, or G^tBA
R = R1, R2, R3, R4, or R5, where
R1 = -CnH2n+1 , n = 2-22, linear or branched
R2 =
R3 = n = 2 - 12
R4 = n = 2 -12
R5 = n = 0 -10

Fig. 1

Compound:	1.15	1.12	1.10	1.8	1.6	1.4	1.17	
Time:	0.60	0.60	0.60	0.60	0.60	0.60	0.60	mins.

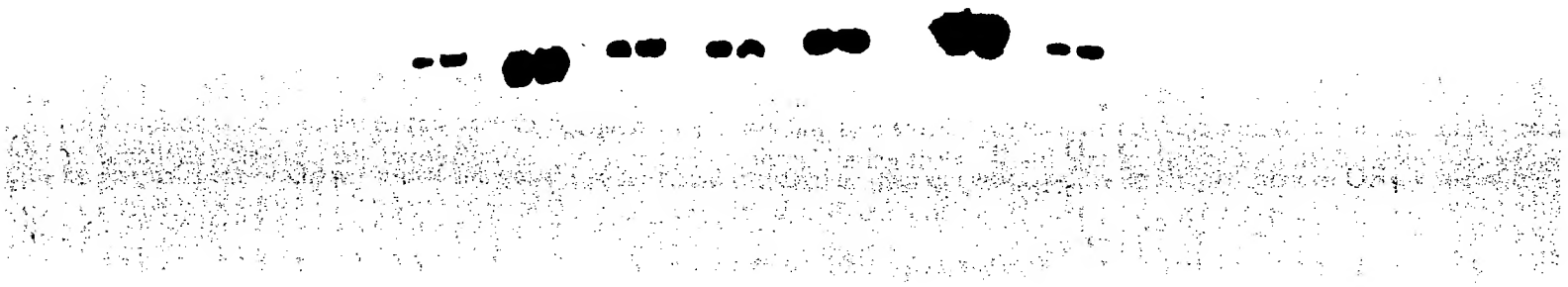


FIG. 2

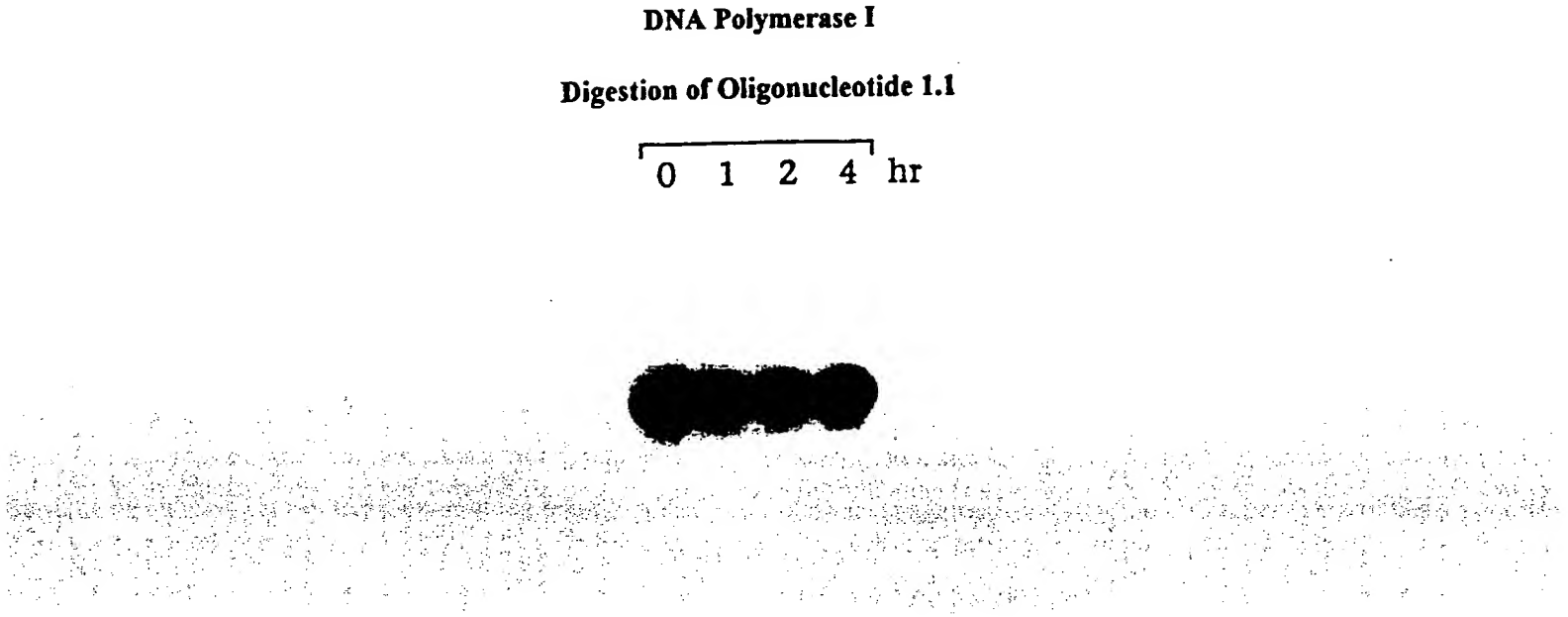


FIG. 3

T4 Polymerase Digesti n of Oligonucleotide 1.0 and 1.1

Compound:	1.0	1.1	
Time:	0 30	0 15 30 60	mins.

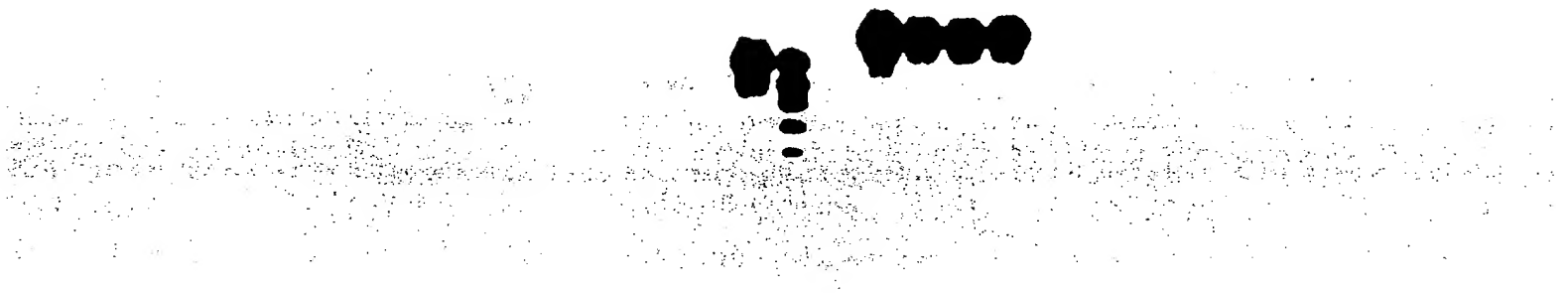


FIG. 4

INTERNATIONAL SEARCH REPORT

Inte nal Application No
PC1/US 96/03843

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H21/00 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 15946 (HYBRIDON, INC.) 21 July 1994 see example 11 ---	1,2
X	WO,A,94 02499 (HYBRIDON, INC.) 3 February 1994 see the whole document --- -/--	1-4,8-20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 August 1996	13.08.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Scott, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/03843

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NUCLEIC ACIDS RESEARCH, vol. 14, no. 18, 1986, OXFORD GB, pages 7405-7420, XP002008077 K.A.GALLO ET AL.: "Alkyl Phosphotriester Modified Oligodeoxyribonucleotides. V. Synthesis and Absolute Configuration of Rp and Sp Diastereomers of an Ethyl Phosphotriester Modified EcoRI Recognition Sequence, d(GGAA(Et)TTCC). A Synthetic Approach to Regio- and Stereospecific Ethylation-Interference Studies." see page 7408, line 13 - page 7409, line 2 ---	1,2
X	NUCLEOSIDES AND NUCLEOTIDES, vol. 8, no. 2, 1989, pages 185-200, XP002008078 M.KOZIOLKIEWICZ ET AL.: "Phosphate-Modified Oligonucleotides." see the whole document ---	1,2
X	NUCLEIC ACIDS RESEARCH, vol. 14, no. 22, 1986, OXFORD GB, pages 9081-9093, XP002008079 L.A.LAPLANCHE ET AL.: "Phosphorothioate-Modified Oligodeoxyribonucleotides. III. NMR and UV Spectroscopic Studies of the Rp-Rp, Sp-SP, and Rp-Sp duplexes d(GGsAATTCC)2, Derived from Diastereomeric O-ethyl Phosphorothioates." see the whole document ---	1,2
A	WO,A,91 04983 (UNIVERSITY PATENTS INC.) 18 April 1991 see the whole document ---	1
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 86, no. 17, September 1989, WASHINGTON US, pages 6553-6556, XP002008080 R.L.LETSINGER ET AL.: "Cholesteryl-Conjugated Oligonucleotides: Synthesis, Properties, and Activity as Inhibitors of Replication of Human Immunodeficiency Virus in Cell Culture." cited in the application see the whole document -----	1,5,6

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/03843

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 15-20
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 15-20 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

formation on patent family members

Inter nal Application No
PCT/US 96/03843

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9415946	21-07-94	AU-B-	6024394	15-08-94
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		US-A-	5218103	08-06-93